(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 867 114 A1

(12) EUROPEAN PATENT APPLICATION

published in accordance with Art. 158(3) EPC

(43) Date of publication:
30.09.1998 Bulletin 1998/40

(21) Application number: **96932037.3**

(22) Date of filing: **27.09.1996**

(51) Int. Cl.[6]: **A01K 67/027**

(86) International application number:
PCT/JP96/02828

(87) International publication number:
WO 97/11597 (03.04.1997 Gazette 1997/15)

(84) Designated Contracting States:
AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **29.09.1995 JP 275110/95**

(71) Applicants:
- **Hoechst Marion Roussel, Ltd.**
  Tokyo 107-8465 (JP)
- **Ogawa, Shyoso**
  Tokyo 156 (JP)
- **Iwaya, Makoto**
  Tokyo 154 (JP)

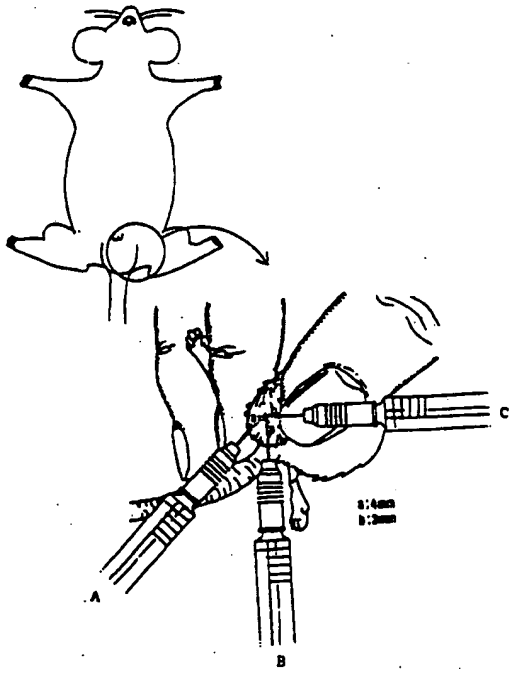
(72) Inventors:
- **OGAWA, Shyoso**
  Tokyo 156 (JP)
- **IWAYA, Makoto**
  Tokyo 154 (JP)
- **SATOH, Masahiro**
  Kanagawa 259-11 (JP)
- **TADA, Norihiro**
  Saitama 336 (JP)

(74) Representative:
Macchetta, Francesco et al
GRUPPO LEPETIT S.p.A.
Patent and Trademark Department
Viale Gran Sasso, 18
20131 Milano (IT)

(54) METHOD FOR PREPARING TRANSGENIC ANIMAL

(57) A method for preparing a sperm containing an exogenous DNA, wherein the exogenous DNA together with liposome for the purpose of transduction of DNA into mammalian cells is injected repeating three times or more into a testis of a mature non-human vertebrate male and the sperm containing the exogenous DNA is produced in the testis of said male. It can be convenient and efficient to prepare a non-human transgenic vertebrate by mating a sperm containing an exogenous DNA with a female naturally, or mating an egg which has not yet been fertilized by artificial insemination or *in vitro* fertilization.

Fig. 2



EP 0 867 114 A1

Detailed Description of the Invention

An object of the present invention is to provide a convenient and efficient method for preparing transgenic animals whereby a large number of transgenic animals may be prepared within a short period, but which does not require a conventional microinjection method. It is expected that, through this invention, it will be possible to solve the hitherto difficult problem of obtaining large transgenic domestic animals, such as cattle.

The present invention relates to a method for preparing an exogenous DNA together with liposome for injecting into a testis of a mature non-human vertebrate male and then producing a sperm containing said exogenous DNA in a testis of said male.

Moreover, the present invention relates to a method for preparing a transgenic non-human vertebrate animal, wherein an exogenous DNA is injected into a test is of a mature non-human vertebrate male after forming a complex with liposome for the purpose of transduction of DNA into mammalian cells, and a sperm is produced in a testis of said male, and then said male is allowed to mate with a female of which egg has not been fertilized yet.

The method of this invention is characterized by mixing an exogenous DNA together with liposome for the purpose of transduction of DNA into mammalian cells, and injecting it directly into a tesits of a mature non-human vertebrate male.

In this specification, "exogenous DNA" refers to "plasmid DNA." The type of an exogenous DNA that is introduced can be changed in order to prepare a desired transgenic animal. Liposome, which is formed to a complex by mixing with an exogenous DNA, can be any commerically available liposome sold for the purpose of transduction of DNA into mammalian cells (cationic liposome). For these liposome, liposome which contains LIPOFECTIN™ : N-[1-(2,3-dioleyloxy)propy]-N,N,N-trimethylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE) at a ratio of 1:1 (w/w) is preferably used. Liposome such as TransIT™-LT1 MIR2310, TransIT™-LT2 MIR2320, TransIT™-LT100 MIR2100 (Takara Shuzo), Genetransfer 074-03621, TRANSFECTAM 561-36591 (Wako Pure Chemical Industries, Ltd.) can be also used. Forming to a complex with an exogenous DNA and liposome is carried out according to a conventional method; for example, an exogenous DNA is added to a solution containing liposome, mixed and incubated.

A liposome-exogenous DNA complex is directly injected into a testis of mature non-human vertebrate male. The number of injection is carried out preferably three times or more. The mature males are anesthetized and the solution containing liposome described above is injected directly from the scrotum into both testes. The injection procedure is repeated at four day intervals for at least three times. On day 2 from the final injection of the liposome-exogenous DNA complex, a sperm containing the exogenous DNA is produced in the testis of said male.

A transgenic animal can be prepared by mating said males with females of whom estrus has been induced by gonadotropic hormone, making the females pregnant and delivered.

A transgenic animal can be prepared by artificial insemination or *in-vitro* fertilization using a sperm produced by the test is of a mature non-human vertebrate male that has been subjected to intratesticular injection of the exogenous DNA and by making the females pregnant and delivered.

The method of the present invention can be applied to efficiently produce non-human vertebrate animals such as experimental animals; a mouse, a rat, a rabbit, a dog, and a cat, etc. as well as domestic animals; a horse, a cattle, a pig, a goast and a sheep, etc.

A transgenic animal obtained by the method of the present invention can be raised and bred under normal conditions of animal husbandry and using ordinary animal feed.

Whether a fertilized egg or an animal obtained contains the exogenous DNA or not is confirmed by the following experiment as illustrated.

Some of the females are sacrificed on the day when copulation is confirmed (determined as the first day of pregnancy), and the intrauterine sperm and the fertilized eggs (single-cell embryos) in the oviduct are sampled. Said samples are analyzed for the presence of an exogenous DNA. The other females are allowed to live until midjestational stages through pregnancy or allowed to come to term. For those allowed to live until midjestational stages through pregnancy, the fetus is extirpated, the DNA extracted, and the rate of incorporation of an exogenous DNA in the fetal chromosome is determined by Southern blotting (Southern, *J Mol Biol.*, 98:503-517, 1975) or PCR method (Saiki *et al.*, *Science*, 239:487-491, 1988). In newborn individuals, the presence of an exogenous DNA is determined from DNA extracted from the tail on the fifth day after birth.

Mature transgenic individuals (referred to as a founder or F0 generation) are allowed to mate with normal animals to produce offspring. DNA from the second generation (F1) individuals is analyzed by Southern blotting or PCR method to determine whether an exogenous DNA has been transmitted from F0 to F3.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a plasmid map for the plasmid pMT-neo/MT-beta-gal, used for DNA transduction.
This has the DNA sequence coding for mouse metallothionein-I promoter (MT), the gene for neomycin resistance

DNA. Approximately 5 μg of the linearized DNA was mixed at a 1:1 ratio with Lipofectin™ (GIBCO-BRL, dissolved in PBS buffer, pH 7.2) at room temperature, and this was allowed to stand for 15 minutes. The manufacturer's instructions were followed for this procedure. In this process the exogenous DNA (pMT-neo/MT-beta-gal) becomes surrounded by a lipid layer of liposome and forms a so-called liposome-exogenous DNA complex. A control solution was prepared by mixing liposome at a 1:1 ratio with PBS.

The liposome-exogenous DNA complex and the control solution were each drawn up using a syringe with 1/6 gauge needle and approximately 20 μl were injected directly through the scrotum into both testes of anesthetized adult ICR male mice (age: 8-10 weeks, obtained from CLEA Japan, Inc.). A schematic drawing of the injection procedure is shown in Fig. 2. After injection of the liposome-exogenous DNA complex, the males were allowed to mate with ICR females (age: 8-10 weeks, obtained from CLEA Japan, Inc.; estrus was induced by gonadotropic hormones) at the second day after DNA injection. Then fertilized eggs (1-cell-embryos) and the intrauterine sperms were recovered from the oviduct and the uterus, respectively. The cumulus oophorus cells were removed from the recovered fertilized eggs by hyaluronidase and were then cultured for three days at 37°C in an atmosphere of 5% CO_2/95% air, on 100 μl of M16 culture fluid (Whittingham, *J. Reprod. Fert.*, 14(Supple):7-21, 1971) containing 1 μM $CdCl_2$ (for the purpose of increasing MT activity), until blastocysts had formed. Then DNA analysis by PCR method was used to determine the presence of an exogenous DNA on the cultured blastocysts. Also, some of the blastocysts were analyzed histochemically for beta-gal activity to determine if the introduced gene was being expressed. The method of Saiki *et al.* (*science*, 239:487-491, 1988) was used for DNA analysis by PCR method. At this time, two primer sets were used: the first comprised the MI499 beta-gal (SEQ ID NO.: 1) and the MI500 beta-gal (SEQ ID NO.: 2), both of which recognize the region on the 3' end of the beta-gal gene (*Molecular Cloning---A Laboratory Manual*, CSH, NY, 1982); the second comprised the MI1511 neo (SEQ ID NO.: 3) and the MI1512 neo (SEQ ID NO.: 4), both of which recognize the region on the 5' end of the neo gene (*Gene*, 19:327-336, 1982). PCR reaction conditions were as follows: 1 μl (approximately 1 μg) of genomic DNA or solution containing blastocysts was added to 9 μl of reaction mixture (10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% (w/v) gelatin, 200 μM dATP, dTTP, dGTP and dCTP, 50 pM of each of primers, and 0.1 μl of Taq polymerase), and this was reacted for 36 PCR cycles. After denaturing for one minute at 95°C, the reaction mixture was annealed at 58°C for one minute, followed by an extension reaction at 75°C for four minutes. The reaction mixture was then subjected to electrophoresis on 4% agarose gel, and the gel was stained with ethidium bromide. After staining, the presence of the amplified exogenous DNA was determined under an ultraviolet illumination.

Histochemical analysis of beta-gal was conducted as follows: specimens (blastocysts) were fixed at room temperature for five minutes in an intimate mixture of 1.25% glutaraldehyde in PBS, then washed three times in droplets of 50 μl PBS containing 0.05% bovine serum albumin (BSA). Subsequently the specimens were reacted for approximately 24 hours at 37°C in PBS (50 μl) containing 1.2 mM 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal), 1 mM $MgCl_2$, 0.1% Triton X-100, 3 mM $K_4Fe[CN]_6 \cdot 3H_2O$ (P3289; Sigma), and 3 mM $K_3Fe[CN]_6$ (P3667; Sigma). This operation causes blue cytoplasmic staining of specimens that exhibit beta-gal activity. No staining occurs in cytoplasm of specimens that exhibit no beta-gal activity.

The recovered intrauterine sperms were subjected to a procedure to isolate genomic DNA. The method of Sato *et al.* (*Animal Biotech*, 5:19-31, 1994) was used to isolate the genomic DNA. Specifically, the intrauterine sperms deposited by light centrifugation was placed in 700 μl lysis buffer (150 μg/ml proteinase K (No. 24568, Merck), 0.5 mg/ml Pronase E (Kaken Kagaku Company), 100 mM NaCl, 0.4% SDS, 10 mM Tris buffer, 10 mM EDTA, pH 8.0), and allowed to belysed at 37°C for 24-48 hours. Then 100 μl phenol was added to the lysis mixture and the supernatant was extracted. The supernatant was then treated at 37°C for 30 minutes in 80 μg/ml ribonuclease A (RNase A), thus decomposing the RNA present in the mixture. This supernatant was then treated with 700 μl of isopropanol, yielding the DNA as precipitate. The DNA precipitate was wiped up using a Gilson Yellow tip, washed in 70% ethanol, and dissolved in 70 μl of TE buffer (10 mM Tris buffer and 1 mM EDTA, pH 7). The genomic DNA derived from intrauterine sperms was subjected to PCR analysis using two different primer sets to determine the presence of exogenous DNA.

Control solution or solution containing a liposome-exogenous DNA (pMT-neo/MT-beta-gal) was injected into the test is of male animals and these mice were then mated with estrus females in the second day after DNA injection, in order to isolate fertilized eggs. Analysis of beta-gal expression was then performed using blastocysts derived from these fertilized eggs (first injection). Four days after the first injection of the liposome-exogenous DNA, the test is was injected a second time with control solution or with the liposome-exogenous DNA. Two days after the second injection of the liposome-exogenous DNA, the males were mated with estrus females. The fertilized eggs were then isolated and the genomic DNA was subjected to DNA analysis and expression analysis, as was done for the group which had received only one injection. Furthermore, four days after the second testicular injection of DNA, another injection of liposome-exogenous or control solution was performed (third injection). Generally the first injection was made from the direction labeled "A" in Fig. 2, with the syringe inserted from the surface of the scrotum to a depth of approximately 4 mm before the complex was injected. The second injection was made from direction labeled "B" in Fig. 2, with the syringe inserted from the surface of the scrotum to a depth of approximately 4 mm before the complex was injected. The third injection was made from direction labeled "C" in Fig. 2, with the syringe inserted from the surface of the scro-

not observed in lane C, which shows the results of PCR analysis of genomic DNA from the tail of a normal mouse. The results are summarized below.

(1) The presence of the exogenous DNA was determined in PCR analysis of genomic DNA in 4 out of 14 embryos (28.6%) that were examined in the experimental group. These results are similar to the results of PCR analysis using primer sets that recognize the neo gene.

(2) Out of 53 embryos examined in the experimental group, 18 (34.0%) were determined to show beta-gal activity.

(3) No exogenous DNA was present in any out of 10 embryos examined in the control group. Nor did any out of 21 embryos in the control group examined for beta-gal activity exhibit such activity.

These results demonstrate that the invented method successfully yielded transgenic embryos that had conserved the exogenous DNA.

EXAMPLE 4 Transmission of the exogenous DNA to the successor generation (F1) from the transgenic individual (F0) sired by a male subjected as an adult to intratesticular injection of liposome-exogenous DNA complex

Since it had been confirmed that the exogenous DNA was present in individuals grown from ova fertilized by sperms that had been subject to phenotypical transformation through intratesticular injection of liposome-exogenous DNA complex, further tests were conducted to determine whether said exogenous DNA would be transmitted to succeeding generations or not.

First, liposome-exogenous DNA complex or control solution was injected three times into ICR male mice, who were then mated with estrus ICR females (8-10 weeks old, estrus was induced by gonadotropins) two days after the final injection. Samples were taken from the tails of offspring of this mating (F0) on the fourth week after birth, and subjected to DNA analysis by PCR method and Southern blotting. The method used to isolate the genomic DNA from the tail and the method of PCR analysis were identical to the same methods described in Example 2. The Southern blotting basically followed the method of Sato *et al.* (*Mol. Reprod. Develop.* 34:349-356, 1993). Specifically, 10 μg of genomic DNA was digested by *Eco*RI and *Bam*HI restriction endonucleases, and then placed on a 0.8% agarose gel. The DNA on the gel was transferred to a nylon membrane filter (GeneScreenPlus™; NEN Company, USA) under alkaline conditions. The nylon membrane filter was then subjected to Southern hybridization using a ^{32}P-labeled probe (DNA fragment from part of the neo gene or beta-gal gene). After Southern hybridization the filter was washed once in 0.1 x SSC/0.01% SDS for 30 minutes at 56°C and exposed tog Kodak XAR-5 film.

The results are shown in Table 2. In the experimental group, the presence of the exogenous DNA was determined in 25 out of 47 F0 individuals (53.2%). This indicates that the exogenous DNA that had been injected into the testis was transmitted *via* the sperms to the offspring.

The second generation of offspring (F1) produced by the F0 transgenic animals was also examined for the presence of the exogenous DNA. Genomic DNA was extracted from the tails of F1 mice according to the procedure described in Example 2. The genomic DNA was subjected to PCR analysis and Southern blotting according to the methods described in Example 2 and Example 4. The partial results are shown in Fig. 7. In Fig. 7, the individuals having the exogenous DNA have been traced using PCR method on genomic DNA extracted from the tail on four-week-old individuals in F0 and F1 generations. Analysis of DNA in F0 descendants demonstrated that approximately 40% of F0 descendants were transgenic. Analysis of DNA from F1 descendants demonstrated that approximately 38% of F1 descendants were transgenic. This proportion indicates that the genomic DNA was probably transmitted to descendants in Mendelian fashion, which is exactly identical to the inheritance of the exogenous gene in the transgenic individuals obtained by the prior art method (microinjection).

Table 1

Gene expression of exogenous DNA in blastocysts sired by males subjected to intratesticular injection of exogenous DNA				
Number of injections of exogenous DNA	(x1)	(x2)	(x3)	Control (x3)
Number of 1-cell embryos recovered after mating, following intratesticular injection of exogenous DNA	82	81	21	40
Number of embryos developed to the two-cell stage (%)	82 (100)	81 (100)	20 (95.2)	40 (100)

paring such animals is a microinjection method by using a micromanipulator to inject an exogenous DNA into a pronucleus of a fertilized egg. However, a certain problems are encountered; this method is time-consuming, the number of treatment at once is limited, the operation requires specialized skills and it requires the use of an expensive micromanipulator.

By using the present method, an exogenous DNA can be injected into 100,000 or more sperm cells at once, which is extremely effective for a domestic animal of which egg is hard to handle by microinjection. Moreover, the present invention is industrially applicable to prepare a trangenic animal simply because a lot of sperm cells containing an exogenous DNA are subject to freeze and thaw for use at a more convenient time, and lend themselves to the use of artificial insemination or *in vitro* fertilization.

SEQ ID NO: 3:

SEQUENCE LENGTH: 26

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE: none

ORGANISM: none

STRAIN: none

FEATURE: primer for recognizing the nucleotides from numbers 1752 to 1767 on the neo gene; called "MI1511 neo"

SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCGTGGCTGG CCACGACGGG CGTTCC

SEQ ID NO: 4:

SEQUENCE LENGTH: 16

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE: none

ORGANISM: none

STRAIN: none

FEATURE: primer for recognizing the nucleotides from numbers 1846 to 1861 on the neo gene; called "MI1512 neo"

SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GACAGGAGAT CCTGCC

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(vi) ORIGINAL SOURCE:
(A) ORGANISM: none
(B) STRAIN: none

(ix) FEATURE:
(D) OTHER INFORMATION:/note= "primer for recognizing the nucleotides from numbers 4278 tc 4298 on the beta-gal gene; called "M1500 beta-gal""

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TACTGACGGG CTCCAGGAGT C 21

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(vi) ORIGINAL SOURCE:
(A) ORGANISM: none
(B) STRAIN: none

(ix) FEATURE:
(D) OTHER INFORMATION:/note= "primer for recognizing the nucleotides from numbers 1752 to 1767 on the neo gene; called "M1511 neo""

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCGTGGCTGG CCACGACGGG CGTTCC 26

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(D) OTHER INFORMATION:/note= "primer for recognizing the nucleotides from numbers 1846 to 1861 on the neo gene; called "M1512 neo""

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GACAGGAGAT CCTGCC 16

SEQ ID NO: 3:

SEQUENCE LENGTH: 26

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE: none

ORGANISM: none

STRAIN: none

FEATURE: primer for recognizing the nucleotides from numbers 1752 to 1767 on the neo gene; called "MI1511 neo"

SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCGTGGCTGG CCACGACGGG CGTTCC

SEQ ID NO: 4:

SEQUENCE LENGTH: 16

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE: none

ORGANISM: none

STRAIN: none

FEATURE: primer for recognizing the nucleotides from numbers 1846 to 1961 on the neo gene; called "MI1512 neo"

SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GACAGGAGAT CCTGCC

Fig. 1

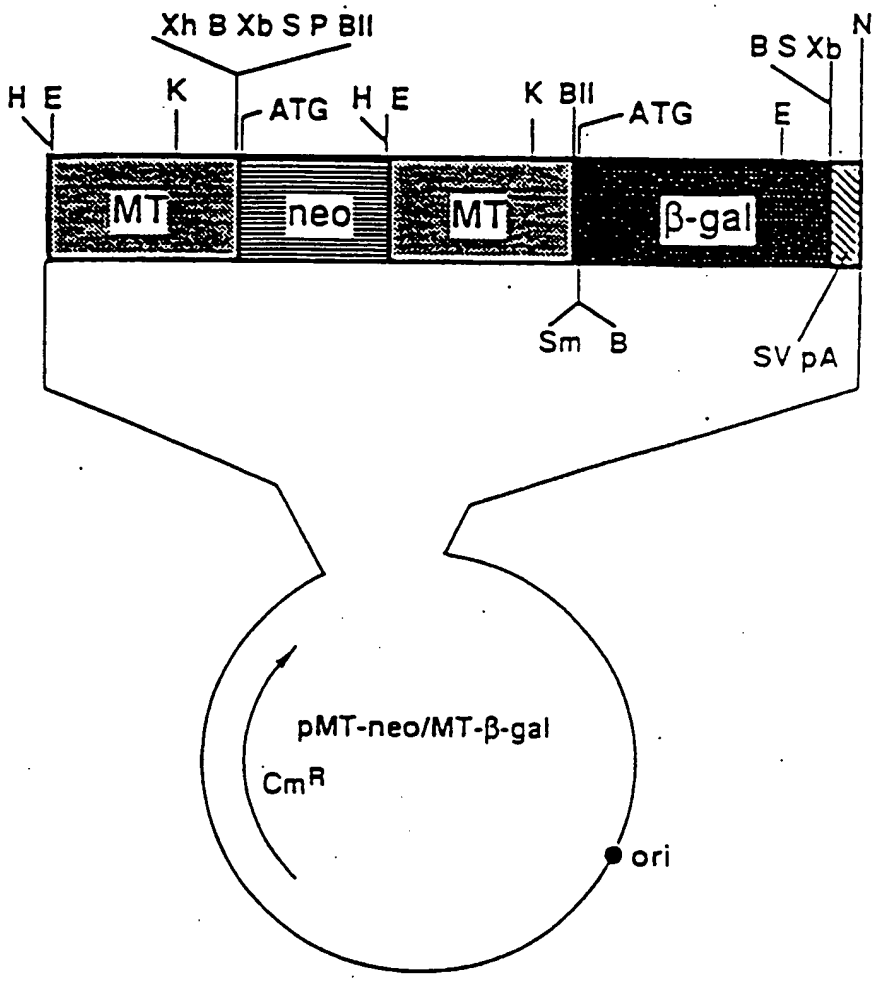

Xh B Xb S P BII
B S Xb
N
H E
K
ATG
H E
K BII
ATG
E
MT
neo
MT
β-gal
Sm B
SV pA
pMT-neo/MT-β-gal
Cm^R
ori

Fig. 3

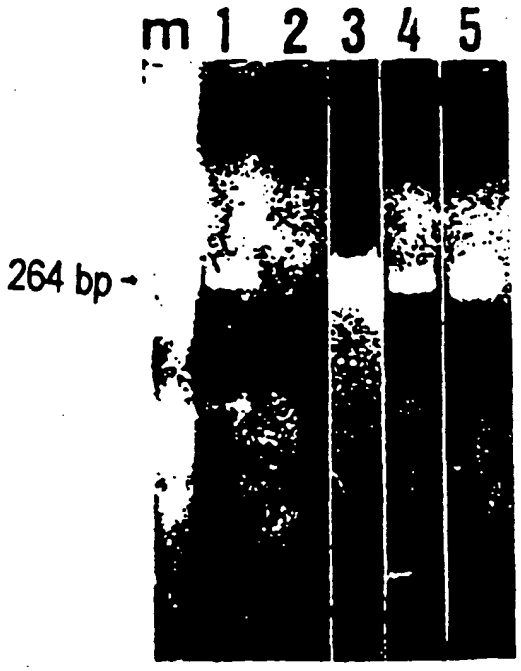


Fig. 5

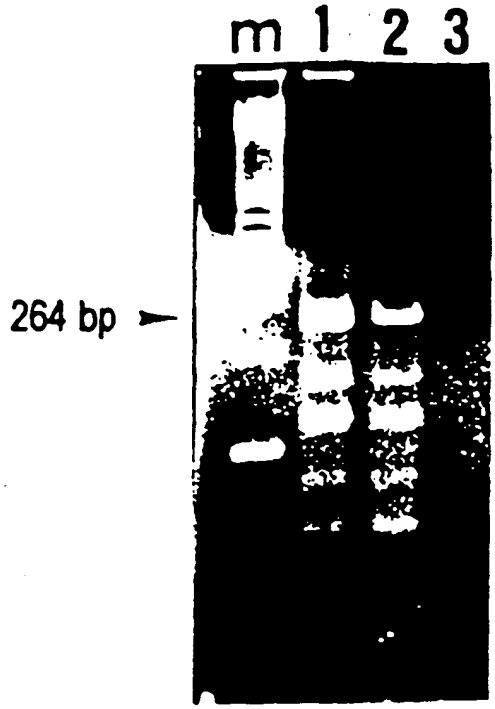
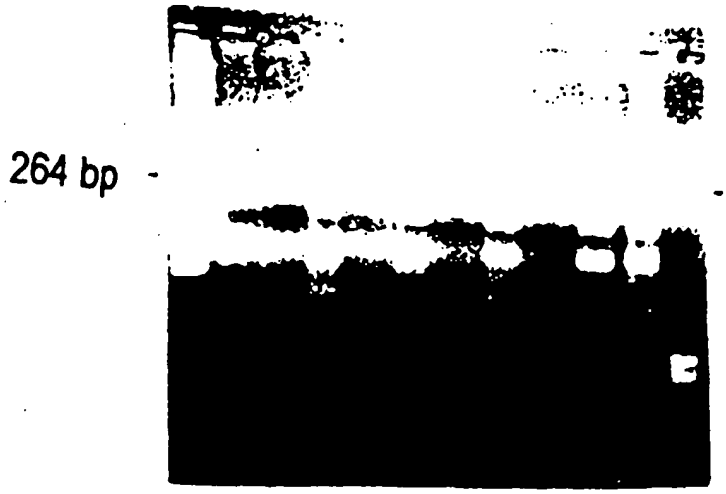


Fig. 6



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP96/02828

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl^6 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl^6 A01K67/027

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Animal Biotech. Vol. 5, No. 1 (1994), p. 19-31 especially p. 27, M. Sato et al. "Direct injection offoreign DNA into mouse testis as a possible alternative of sperm-mediated gene transfer"	1 - 8
A	Science Vol. 261 (1993), p. 209-211, Zhu N. et al. "Systemic gene expression after intravenous DNA delivery into adult mice"	1 - 8
A	Mol. Reprod. Dev. Vol. 30 (1993), p. 194-200, Bachiller D. et al. "Liposome-mediated DNA uptake by sperm cells"	1 - 8
A	Proc. Natl. Acad. Sci. USA, Vol. 86 (1989), p. 6982-6986, Behr J.P. et al. "Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA"	1 - 8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
October 9, 1996 (09. 10. 96)	October 22, 1996 (22. 10. 96)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)